Teratogen-induced eye defects mediated by *p53*-dependent apoptosis

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Background: Many birth defects are believed to involve gene–environment interactions, although the mechanisms involved are poorly understood. Apoptosis is a common effect of many kinds of environmental stresses on the developing embryo; therefore, mechanisms of teratogenesis may be approached within the context of the cell death program. The *p53* tumor suppressor gene encodes a transcription factor which functions as a critical regulator of apoptosis in response to environmental stress.

Results: To investigate the relationship between *p53*-dependent apoptosis and teratogenesis, we subjected day 8 mouse embryos with different *p53* gene backgrounds to a genotoxic stress, 2-chloro-2′-deoxyadenosine. Treatment rapidly stimulated nuclear p53 accumulation and triggered apoptosis in some (head-fold) but not other (primitive heart) developing structures. Induced cell death was *p53* gene-dose dependent, as shown by the intermediate sensitivity of 4–5 somite stage embryos bearing only a single effective *p53* allele and the lack of sensitivity of *p53*-null mutants. Abnormal development was manifested as eye defects by day 11, particularly lens agenesis. Overall the incidences of these defects at term were 73.3 % for *p53* wild-type fetuses, 52.5 % for heterozygous mutants, and 2.2 % for *p53*-null mutants. Statistical analysis indicated that the interaction between teratogen and genotype was highly significant ($P \leq 0.001$) for cell death on day 8 and eye defects on day 17.

Conclusions: We conclude that teratogen induction of *p53*-dependent apoptosis in the developing embryo is positively coupled to the determination of congenital eye defects.

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Background

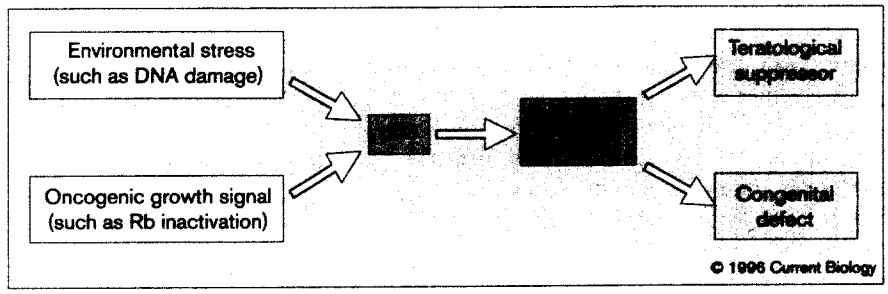
Drugs and chemicals that induce excessive cell death in the embryo often cause congenital malformations in experimental animals [1]. However, the specific contribution of teratogen-induced cell death to the pathogenesis of congenital malformations is difficult to predict because of a remarkable capacity of early embryos to compensate for cell loss [2]. With the recognition that many teratogenic treatments trigger apoptosis [3–6], the genetic circuitry of the cell death program provides an approach to assess the impact of excessive cell death on the pathogenesis of congenital malformations.

One of the important positive regulators of apoptosis is the *p53* tumor suppressor gene. The *p53* gene encodes a transcription factor which is activated by certain kinds of environmental stress [7–10] or oncogenic growth signals [11–13]. Loss of *p53* function facilitates neoplastic development, as shown by the fact that *p53* is mutated in many human tumors [14,15] and that *p53*-deficient mutant mice are predisposed to tumorigenesis [16–18]. Thymocytes from *p53*-null mutant ($p53^{-/-}$) mice resist cytotoxic doses of ionizing radiation [8,9], chemotherapeutic agents [10], and metabolic toxins [19] that ordinarily damage DNA, but these cells retain a normal susceptibility to hormonally induced apoptosis. This has been interpreted as indicating that distinct forms of cell death exist which are *p53*-dependent and *p53*-independent.

Previous studies have shown that *p53*-dependent events may have positive or negative effects on the developing embryo [20–24]. Flooding the blastomeres of *Xenopus* embryos with a wild-type *p53* expression plasmid severely disrupted germ layer formation and resulted in death of the embryo by the onset of neurulation [20]. Overexpression of wild-type *p53*, under the control of the lens-specific αA-crystallin gene promoter, induced excessive cell death in the developing lens and disrupted eye development in transgenic mice [21]. On the other hand, deficiency of *p53* has been associated with spontaneous [22,23] and induced [24] abnormal development. For example, between 8–16 % of $p53^{-/-}$ mutant mouse embryos display a severe defect in anterior neural tube closure, known as exencephaly [22]. Increased incidences

Figure 1

Scenarios for the possible relationship between *p53*-dependent apoptosis and teratogen-induced abnormal development in mammalian embryos.



of fetal malformations, resorptions, and neonatal deaths have been reported among $p53^{+/-}$ heterozygous dams treated with a mildly teratogenic regimen of dioxin and benzo[a]pyrene, compared to similarly treated $p53^{+/+}$ wild-type dams, in litters sired by a $p53^{+/-}$ male [24]. These results were interpreted as indicating that *p53* is a teratological suppressor gene which responds to DNA damage and developmental oxidative stresses; however, the linkage between *p53*-dependent events and teratogen-induced abnormal development currently remains an open question, particularly with respect to the role of cell death (Fig. 1). To clarify this issue, we have investigated the relationship between teratogen-induced cell death and fetal malformation, taking into consideration the *p53* gene background of the conceptus. Our results provide the first direct evidence that *p53*-dependent apoptosis mediates chemical teratogenesis, and that *p53*-dependent events are positively coupled to teratogen-induced eye defects.

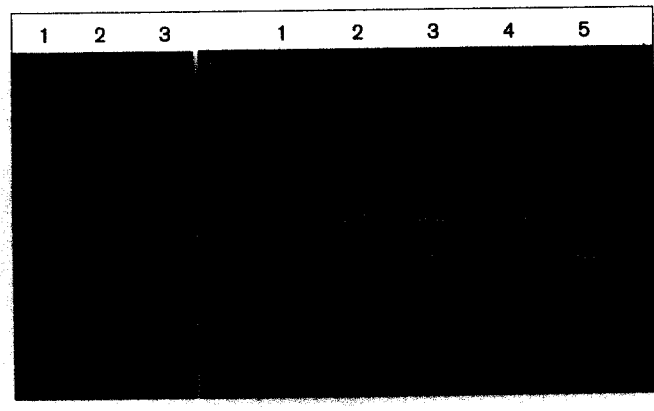
Results and discussion

Deoxyadenylate stress, a model to test *p53* function in early embryos

The viability of early mouse embryos is acutely sensitive to 2′-deoxyadenosine intoxication during gastrulation and neurulation on days 7–8 post-coitus (pc) [25]. Expansion of 2′-deoxyadenosine 5′-triphosphate (dATP) pools is associated with rapid and widespread induction of apoptosis in the neuroectoderm and other tissues of day 8 embryos, but spares the primitive heart [3]. A stable purine analogue, 2-chloro-2′-deoxyadenosine (2-CdA), is a potent DNA-damaging drug which mimics the cytotoxic activity of 2′-deoxyadenosine. Like the natural cognate, 2-CdA readily enters dATP pools of target cells through a process sensitive to Bcl-2 [26] and produces DNA strand breakage. A concentration of 1 μM 2-CdA produces physical strand breaks equivalent to that produced by 1 Gray ionizing radiation [27,28]. Given that such lesions constitute a potent signal for nuclear p53 accumulation [29–31], we reasoned that 2-CdA would be a good prototype teratogen with which to test for *p53* gene function in the day 8 mouse embryo.

To determine the extent of *p53* expression at this early stage of development, northern blot analysis was performed on total cellular RNA of the embryo proper (Fig. 2a). The signal on day 8 pc was equivalent to day 11 pc, a stage already known to represent peak expression of *p53* during mouse embryogenesis [32,33]. Therefore, subsequent experiments were conducted with 2-CdA exposure on day 8 pc, in order to evaluate the following: nuclear p53 accumulation, breakdown of embryonic DNA, apoptosis and structural malformations. The notion that genotoxic agents may induce p53 accumulation is well established [29–31]; furthermore, it is known that apoptosis can be induced by acute treatment with genotoxic agents *in vitro* [7–10] and *in vivo* [34,35], and that this apoptosis is at least partly due to p53-dependent events, because cells from *p53*-null mutant mice are far less susceptible under the same conditions of exposure. To extend these observations to early embryos, we employed the TSG–p53 strain which harbors a deletion of exon 5 in the *p53* gene [16]. The polymerase chain reaction (PCR) was used to identify wild-type ($p53^{+/+}$), heterozygous ($p53^{+/-}$), and nullizygous ($p53^{-/-}$) conceptuses (Fig. 2b).

Figure 2



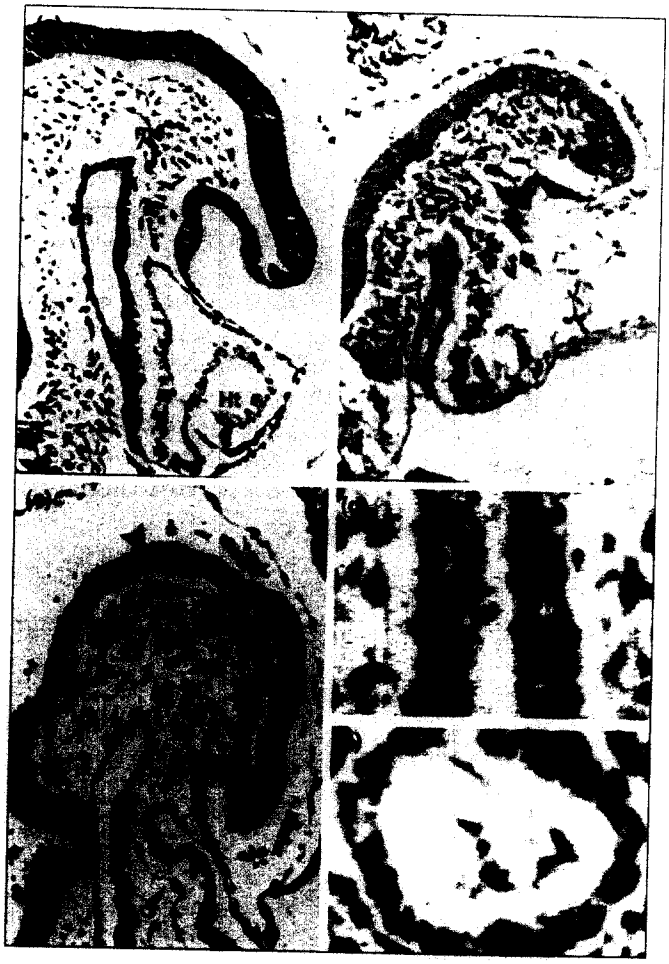
(a) Northern blot analysis of *p53* expression. Total cellular RNA (30 μg per lane) probed with murine *p53* cDNA; lane 1, day 8 pc mouse embryo; lane 2, day 11 pc mouse embryo; and lane 3, adult kidney. Even loading was verified by ethidium-bromide staining. The broad 2.2 kbp band corresponded in size to the murine *p53* transcript. **(b)** Typical results of *p53* allelotyping by PCR for wild-type (1.2 kbp) and targeted (0.6 kbp) alleles: lane 1, *HaeIII* digest of ΦX174 DNA; lane 2, $p53^{+/+}$ fetus; lanes 3 and 4, $p53^{+/-}$ fetuses; lane 5, $p53^{-/-}$ fetus.

Tissue-specific induction of nuclear p53

Day 8 mouse embryos were processed for histology and sections were subjected to immunoperoxidase staining using CM5 antiserum directed against murine p53 [36]. Control embryos displayed relatively uniform immunostaining with little or no signal in the nucleus (Fig. 3a). In view of the high abundance of *p53* transcripts at this stage, the rather unremarkable pattern of p53 immunoreactivity is similar to that of other normal tissues where p53 undergoes rapid turnover [32].

If p53 was responsive to DNA damage at this early stage of development, then exposure of embryos to 2-CdA should result in a demonstrable increase in p53-containing nuclei.

Figure 3



Analysis of p53 expression in day 8 mouse embryos. **(a)** Longitudinal section through the head-fold of an untreated $p53^{+/+}$ embryo reacted with CM5 antiserum; ne, neuroectoderm; me, cranial mesenchyme; en, foregut endoderm; Ht, primitive heart. **(b)** Similar field of a $p53^{-/-}$ embryo fixed 4.5 h after 2-CdA exposure. **(c)** $p53^{+/+}$ embryo fixed 4.5 h after 2-CdA exposure; arrowhead, p53-positive nucleus. Magnification, × 75. **(d,e)** Magnification of cranial neural folds (d) and primitive heart (e) of a $p53^{+/-}$ embryo 4.5 h after 5 mg kg^{-1} 2-CdA treatment. Magnification, × 225.

This prediction was tested by treating pregnant dams with 5 or 10 mg kg^{-1} 2-CdA on day 8 pc. Embryos were processed for immunostaining at 1.5, 3, or 4.5 h post-treatment, based on the previous study which showed that apoptosis related to 2′-deoxyadenosine intoxication peaks within 4.5 h [3]. Nuclear p53 immunoreactivity was first demonstrable in the embryo at 3 h post-treatment, and a striking increase was evident at 4.5 h. Many embryonic tissues displayed positive nuclear p53 immunoreactivity. The most dramatic response overall occurred in the neuroectoderm of the head-fold region (Fig. 3c) and the trophoblast of the ectoplacental region (data not shown).

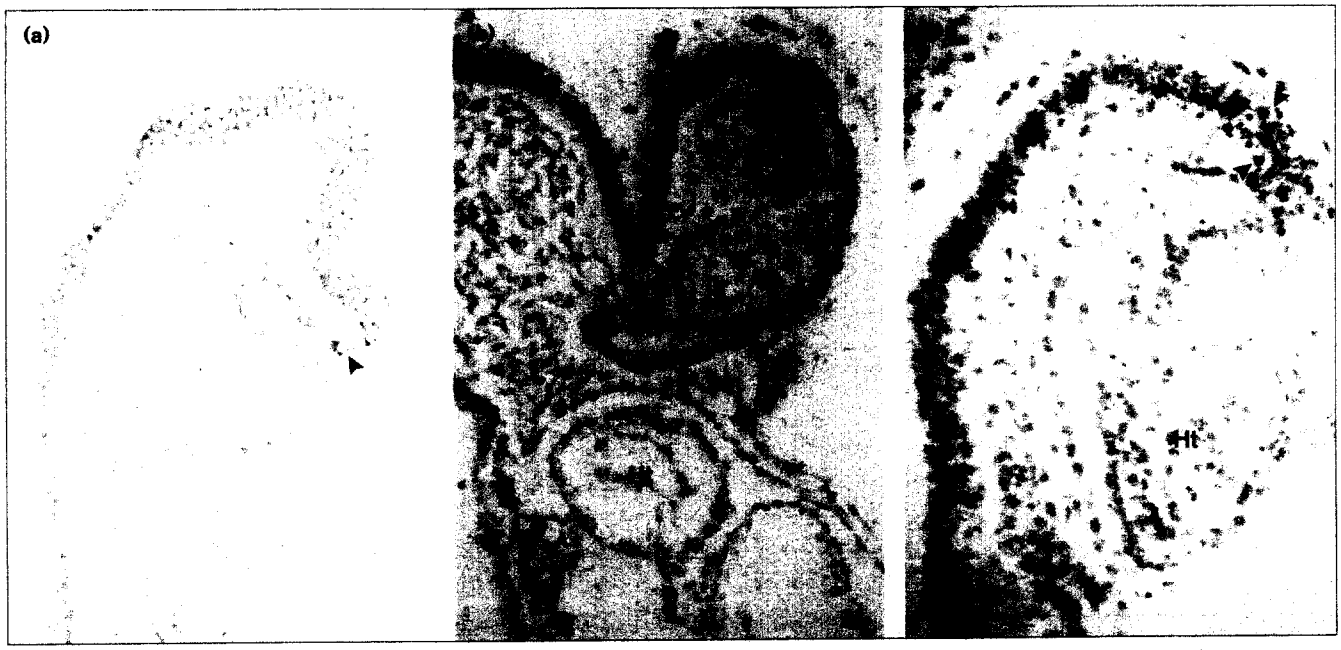
The specificity of nuclear immunoperoxidase staining was verified by the absence of nuclear immunoreactivity in sections from $p53^{+/+}$ embryos treated with 2-CdA and subjected to immunoperoxidase staining using non-immune serum in place of CM5 antiserum (data not shown), and in nuclei from $p53^{-/-}$ embryos treated with 2-CdA and immunostained with CM5 antiserum (Fig. 3b). Concerning the latter, the expression of a truncated mutant p53 protein is not expected in TSG–p53 null mutant mice [16,36] and so the weak signal in these histological sections may be attributed to nonspecific staining [29]. Not all tissues displayed a p53-positive nuclear phenotype following 2-CdA exposure. In particular, the primitive heart did not show p53 induction, whereas the corresponding cranial neuroectoderm showed a dramatic response (Fig. 3d,e). We conclude that nuclear p53 accumulation is induced in a tissue-specific manner by acute exposure of early embryos to a DNA-damaging agent.

The tissue specificity of p53 induction has been reported previously in adult murine tissues following whole body irradiation with 5 Gy γ radiation and immunostaining with the CM5 antiserum [36]. In that study, some cell types (splenocytes, thymocytes, and osteocytes) displayed dramatic accumulation of p53 protein, whereas others (hepatocytes) did not. Because apoptosis accompanied p53 induction in some cell types (splenocytes, thymocytes) but not others (osteocytes), it was concluded that tight control exists with respect to upstream initiation signals and downstream consequences of p53 induction in adult tissues [36]. Perhaps the differential regulation of similar controls in developing embryos contribute to the mechanism of susceptibility to teratogenic effects. Such differences may explain why organ systems become selectively vulnerable to chemical treatment at specific stages of development.

Tissue-specific induction of *p53*-dependent apoptosis in embryos

Physical DNA strand breakage is necessary and sufficient to induce nuclear p53 accumulation during environmental stress [7,30,31]. In these experiments, the breakdown of embryonic DNA was monitored by the terminal deoxynucleotidyl transferase-mediated dUTP–digoxygenin

Figure 4



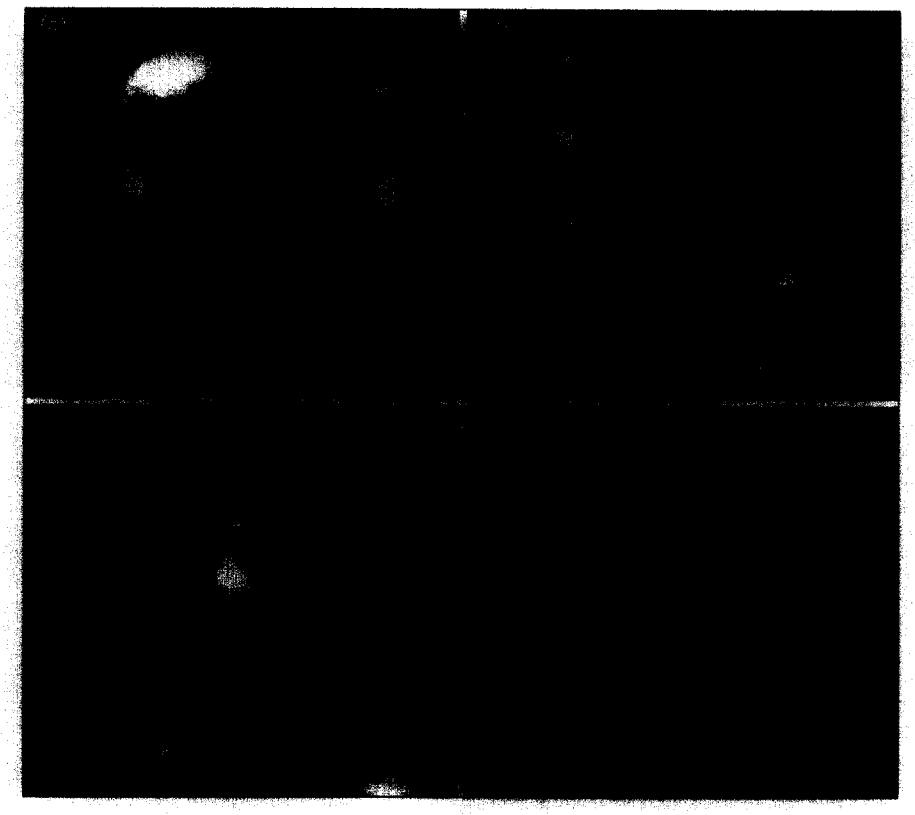
Distribution of TUNEL-positive nuclei in day 8 mouse embryos. **(a)** Untreated $p53^{+/+}$ embryo displaying TUNEL-positive cells (brown/purple) in the rostral extent of the forebrain (arrowhead); counterstain with methyl green. **(b)** $p53^{+/-}$ embryo 4.5 h after 2-CdA exposure. **(c)** $p53^{-/-}$ embryo 4.5 h after 5 mg kg^{-1} 2-CdA exposure; arrowheads denote examples of TUNEL-negative cellular degeneration in the neuroectoderm. Ht, primitive heart; en, foregut endoderm; ne, neuroectoderm. Magnification, × 110.

nick-end labeling (TUNEL) method [37]. In control embryos, DNA fragmentation detected by TUNEL was restricted to naturally occurring regions of programmed cell death, such as the rostral prosencephalon (Fig. 4a). When $p53^{+/+}$ or $p53^{+/-}$ embryos were treated with 2-CdA, the number of TUNEL-positive nuclei increased dramatically in the tissues also shown to become positive for nuclear p53 accumulation. For example, cranial neuroectoderm, mesenchyme, and foregut endoderm displayed striking TUNEL-positive nuclei, whereas the primitive heart showed methyl green counterstaining only (Fig. 4b). In contrast, *p53*-null mutant embryos did not show an increase of TUNEL-positive cells following exposure; however, there was evidence of TUNEL-negative cellular degeneration in the counterstain for some specimens, particularly after higher dosages of 2-CdA (Fig. 4c). TUNEL-negative degeneration was *p53*-independent as demonstrated by its occurrence in all treated embryos irrespective of *p53* gene background. Superimposition of *p53*-dependent and *p53*-independent forms of cell death have been described in other examples of cytotoxicity [10].

Nuclear p53 accumulation could affect cells in various ways, depending on their lineage and state of differentiation, with apoptosis being one possible fate [7,30,31,34,35]. To verify that TUNEL-positive labeling represented apoptosis, embryos were directly exposed to 2-CdA in whole embryo culture and analyzed by acridine orange (AO) fluorescence staining [3]. We used a whole embryo culture system to ensure that embryos were evenly exposed to 5 μM 2-CdA; these embryos were age-matched at the time of exposure (4–5 somite stage). Treatment induced widespread apoptosis in the head-fold and other tissues, as detected by AO-staining (Fig. 5). In contrast, the primitive heart was not affected (data not shown). This pattern essentially matched that of p53-positive and TUNEL-positive nuclear labeling after intrauterine exposure to 5 mg kg^{-1} 2-CdA. Cultured embryos were classified in three groups based on the AO-staining phenotype of the head-fold (Table 1). Statistical analysis by two-way analysis of variance indicated that the effects of 2-CdA treatment, of the *p53* genotype and, more importantly, of the treatment–genotype interaction, were highly significant ($P \leq 0.001$). Significant differences were localized by the unpaired *t*-test, which showed the following: firstly, 2-CdA triggered excessive cell death in $p53^{+/+}$ and $p53^{+/-}$ embryos, but not in $p53^{-/-}$ embryos; secondly, the effect was stronger in $p53^{+/+}$ than in $p53^{+/-}$ embryos. Clearly, the extent of induced apoptosis in stressed embryos was *p53* gene-dose dependent, as shown by the intermediate sensitivity of embryos bearing only a single effective *p53* allele and the lack of sensitivity of null mutants.

As a rule, DNA-damaging agents induce excessive cell death in the neuroectoderm of early rodent embryos but spare the primitive heart [3,4,6,38]. Analysis of developing

Figure 5



Teratogen-induced apoptosis in 4–5 somite stage mouse embryos visualized by AO-fluorochroming. **(a)** Dorsal view of the head-fold of a $p53^{+/+}$ embryo cultured for 3 h with control medium and displaying a normal (type-1) cell death phenotype; **(b)** similar field of a $p53^{+/+}$ embryo cultured for 3 h in the presence of 5 mM 2-CdA and displaying widespread apoptosis (arrowhead), a type-3 phenotype; **(c)** $p53^{+/-}$ embryo cultured with 5 mM 2-CdA and displaying a type-2 phenotype; **(d)** $p53^{-/-}$ embryo cultured with 2-CdA and displaying a normal (type-1) cell death phenotype. Magnification, × 40.

mouse embryos by *in situ* hybridization during organogenesis has indicated that *p53* transcripts are uniformly abundant in the neuroectoderm but undetectable in the developing heart [33]. Perhaps the higher basal expression of *p53* moves certain cells, such as the neuroectoderm, into a state of readiness for cell death to facilitate the deletion of abnormal or damaged cells from undifferentiated structures [39].

Congenital eye defects are a manifestation of *p53*-dependent apoptosis

The induction of *p53*-dependent apoptosis in the early neuroectoderm raises the question of what, if any, impact this effect would have on subsequent development. Apoptotic death of a small number of cells could facilitate normal development by deleting teratogen-damaged cells before they had an opportunity to interfere with

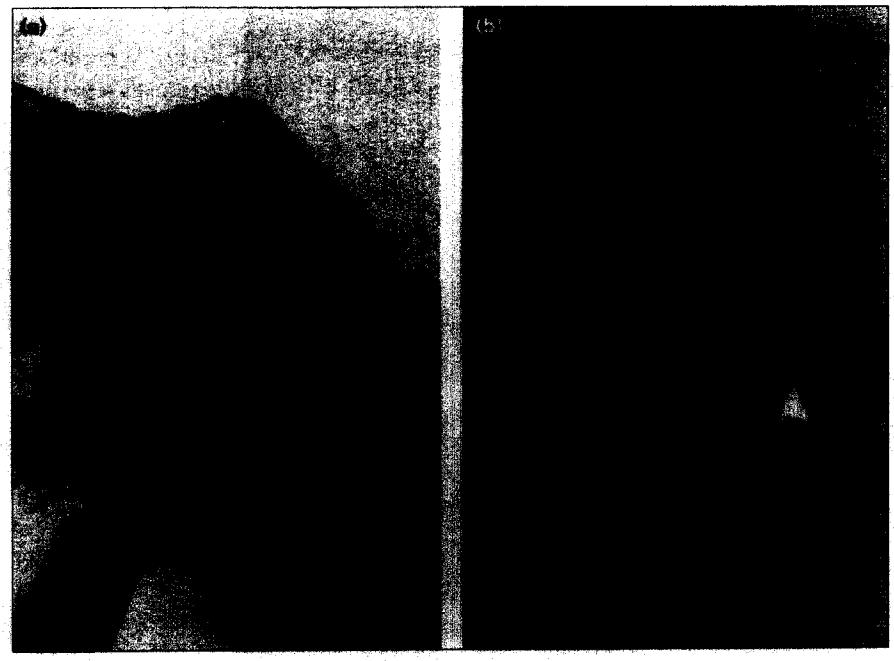
Table 1

Influence of *p53* genotype on induced apoptosis in cultured day 8 embryos.

Embryos*		AO-phenotype†			Analysis‡		
Treatment	Genotype	Type 1	Type 2	Type 3	Index	Treatment Prob *t*	Genotype Prob *t*
Control	$p53^{+/+}$	7	1	0	1.13 ± 0.35	–	–
Control	$p53^{+/-}$	10	1	0	1.09 ± 0.30	–	0.824
Control	$p53^{-/-}$	2	0	0	1.00 ± 0.00	–	0.645
2-CdA	$p53^{+/+}$	0	0	9	3.00 ± 0.00	0.000§	–
2-CdA	$p53^{+/-}$	2	7	1	1.90 ± 0.57	0.001§	0.000§
2-CdA	$p53^{-/-}$	3	0	1	1.50 ± 1.00	0.541	0.001§

*Embryos (4–5 somite stage) were grown for 3 h in whole embryo culture in the presence or absence of 5 μM 2-CdA. †Apoptosis was monitored by AO staining, and embryos were classified as normal (type 1), weak (type 2), or strong (type 3) based on the phenotype of the head-fold (Fig. 5). ‡Statistical analysis of index, determined by assigning numerical values (1, 2 or 3) to the embryos based on AO-phenotypes ± S.D.; effects were highly significant by two-way analysis of variance for treatment, genotype, and treatment–genotype interaction ($P \leq 0.001$); differences (§) were localized by the unpaired *t*-test (Prob *t*).

Figure 6



Analysis of p53-dependent eye defects in day 11 embryos, 3 d after exposure to 5 mg kg^{-1} 2-CdA. Hematoxylin and eosin staining of eyes from **(a)** $p53^{+/-}$ and **(b)** $p53^{-/-}$ littermates; oc, optic cup; arrowhead denotes the lens vesicle which is detached from the surface ectoderm. Magnification, × 75.

morphogenesis. As a putative teratological suppressor gene [24], *p53* would confer a selective survival advantage in the wild-type condition, presuming that the embryo had sufficient plasticity to compensate for the lost cells [2]. In this scenario, *p53*-deficient mutant embryos might show increased incidences of the manifestations of abnormal development (resorption, malformation, intrauterine growth retardation, functional deficits) following teratogenic treatment. On the other hand, the death of an excessive number of cells may disrupt the integrity of specific organ primordia, leading to fetal malformations, the specificity and distribution of which would again depend upon compensatory growth potential (Fig. 1).

To determine the longer term impact of cell death on fetal development, two female $p53^{+/-}$ mice were treated with 5 mg kg^{-1} 2-CdA on day 8 pc and the embryos were examined on day 11 (44–46 somite stage; Theiler stage 19 [40]). Abnormal eyes were grossly apparent in 11 of 15 $p53^{+/+}$ and $p53^{+/-}$ embryos, whereas all three $p53^{-/-}$ siblings in these litters were normal. Early development of the mouse eye involves evagination of optic pits from the diencephalon (day 8), induction of the lens placode by the optic vesicle (day 9), formation of the lens vesicle and optic cup (day 10), and detachment of the lens vesicle from the surface ectoderm (day 11). The histological features of the eyes were examined on day 11 pc in three $p53^{+/-}$ embryos which were overtly abnormal, and three $p53^{-/-}$ embryos which were overtly normal. In one of the $p53^{+/-}$ embryos, both optic cups were severely reduced or missing (data not shown); the other two $p53^{+/-}$ embryos had relatively normal optic cups but lacked organized lens vesicles (aphakia). A typical example of an embryonic eye with aphakia, wherein the lens vesicle has been replaced by a plug of angiogenetic mesenchyme, is shown in Figure 6a. In contrast to the abnormal $p53^{+/-}$ embryos, all three of the $p53^{-/-}$ siblings treated with 2-CdA displayed normal eye histology at Theiler stage 19 (Fig. 6b). We conclude that the developing structure which seemed to be most sensitive to *p53*-dependent dysmorphogenesis was the lens. As the lens vesicle is derived from the surface ectoderm, the precursor population of the head ectoderm may have been deleted by ectopic *p53*-dependent apoptosis. Another possible explanation for failed lens induction could be agenesis of the optic vesicle, again related to ectopic *p53*-dependent apoptosis, but this time afflicting the precursor population of the diencephalic neuroectoderm. Additional studies are needed to establish which of these possibilities, if either, applies to the pathogenesis of lens defects induced by 2-CdA.

To provide a more complete analysis of teratogen susceptibility, pregnant dams were treated with 2-CdA on day 8 pc and the fetuses were evaluated near term (day 17 pc). High incidences of ophthalmopathies were again evident and the types of ocular defects were consistent with the histological analysis on day 11 pc (Fig. 7). A caveat, however, was that 5 mg kg^{-1} 2-CdA induced 49 % fetal resorptions. A previous study [24] reported that $p53^{+/-}$ and $p53^{-/-}$ embryos are more prone to teratogen-induced resorptions than their wild-type counterparts. In our study, fetal resorption did not significantly alter the

Figure 7

Eye defects in near-term fetuses. **(a)** Head of control $p53^{+/+}$ fetus on day 17 pc stained with Alizarin red S to visualize skull bones (red); the normal eye is easily distinguished from the infraorbital foramen by dark pigmentation of the retina. (b–d) Siblings from a litter treated with 5 mg kg^{-1} 2-CdA on day 8 of gestation. **(b)** $p53^{-/-}$ fetus with normal eyes; **(c)** $p53^{+/-}$ fetus with microphthalmia and shortened snout; **(d)** $p53^{+/+}$ fetus with apparent anophthalmia.

expected Mendelian distribution of genotypes near term; furthermore, 10 mg kg^{-1} 2-CdA was fully embryolethal, irrespective of the *p53* gene background of the conceptus. A second round of experiments was performed anyway using a lower dosage of 2-CdA (4 mg kg^{-1}) which did not increase the resorption incidence over the control level. The results of all fetal evaluations are given in Table 2. Eye phenotypes were classified into four groups [41], based on the level of severity of abnormality, with type 1 being normophthalmic and type 4 anophthalmic. In $p53^{+/+}$ fetuses, the incidences of malformed eyes were 46.9 % and 88.8 % for the 4 and 5 mg kg^{-1} 2-CdA dosages, respectively (an overall incidence of 73.3 % malformed eyes). Only one malformed eye was found in the treated $p53^{-/-}$ group (overall incidence of 2.2 % malformed eyes) and this particular individual resembled the spontaneous exencephalic reported in another $p53^{-/-}$ mutant strain [22]. Whether or not the singular eye defect among $p53^{-/-}$ fetuses was caused by the 2-CdA treatment is unknown.

Statistical analysis of these results by two-way analysis of variance indicated that the effects of 2-CdA treatment, of the *p53* genotype and, more importantly, of the treatment–genotype interaction, were highly significant ($P \leq 0.001$). Significant differences were localized by the unpaired *t*-test, which showed the following: firstly, 2-CdA induced dose-dependent ocular malformations in $p53^{+/+}$ and $p53^{+/-}$ fetuses, but not $p53^{-/-}$ fetuses; secondly, the effect was stronger in $p53^{+/+}$ than in $p53^{+/-}$ fetuses. Heterozygotes showed intermediate sensitivity as demonstrated by their relative resistance to induced eye defects at the lower 2-CdA dosage (7.1 % malformed eyes) and the fact that only 1 of 16 anophthalmias produced at the higher dosage sorted out to a $p53^{+/-}$ fetus; the others being wild-type (Table 2). Overall, the incidence of malformed eyes among heterozygotes treated with 2-CdA was 52.5 %. We conclude that the incidence and severity of teratogen-induced eye defects were *p53* gene-dose dependent.

Statistical analysis indicated that the interaction between 2-CdA treatment and embryonic *p53* gene background was highly significant ($P \leq 0.001$) for cell death at the 4–5 somite stage and for eye defects near term. Broadly stated, these data show a highly significant teratogen–gene interaction which involves *p53*-dependent events in the embryo, as assessed by an acute (embryonal cell death) or a late (fetal eye defects) end-point. A plausible hypothesis is that the eye defects were a specific consequence of *p53*-dependent apoptosis; however, induction of the p53 protein, *p53*-dependent DNA strand breaks, and

Table 2

Influence of *p53* genotype on induced ocular malformations in day 17 fetuses.

Fetuses*		Eye phenotype†					Analysis‡			
Dosage	Genotype	Type 1	Type 2	Type 3	Type 4	Total	Index	Treatment Prob *t*	Genotype Prob *t*	Implants resorbed
Control	$p53^{+/+,+/-}$	58	0	0	0	58	1.00 ± 0.00	–	–	7/36 (19 %)
4 mg kg^{-1}	$p53^{+/+}$	17	12	3	0	32	1.56 ± 0.67	0.000§	–	
4 mg kg^{-1}	$p53^{+/-}$	39	2	1	0	42	1.10 ± 0.37	0.052	0.000§	8/60 (13 %)
4 mg kg^{-1}	$p53^{-/-}$	29	1	0	0	30	1.03 ± 0.18	0.166	0.000§	
5 mg kg^{-1}	$p53^{+/+}$	6	17	16	15	54	2.74 ± 0.99	0.000§	–	
5 mg kg^{-1}	$p53^{+/-}$	10	11	26	1	48	2.38 ± 0.84	0.000§	0.049§	53/109 (49 %)
5 mg kg^{-1}	$p53^{-/-}$	10	0	0	0	10	1.00 ± 0.00	–	0.000§	

*Fetuses were exposed on day 8 of gestation and evaluated on day 17; weights were not significantly affected. †Gross morphology of individual eyes (41) were: normal (type 1), colobomas or abnormal pupillary ring (type 2), microphthalmia (type 3), and apparent anophthalmia (type 4); a low incidence of cleft face (9.8 %) and open eyelid (3.9 %) were associated with some $p53^{+/+}$ and $p53^{+/-}$ fetuses, and of exencephaly (10 %) in treated $p53^{-/-}$ fetuses. ‡Statistical analysis of index, determined by assigning numerical values (1, 2, 3 or 4) to the fetuses based on eye phenotypes ± S.D.; effects were highly significant by two-way analysis of variance for treatment, genotype, and treatment–genotype interaction ($P \leq 0.001$); differences (§) were localized by the unpaired *t*-test (Prob *t*).

AO-detected apoptosis also occurred in many regions of the embryo, such as the neural folds, that did not develop abnormally. This shows that *p53*-mediated apoptosis is not universally sufficient to induce teratogenesis.

Eye development is a common morphological marker for experimental teratogenesis. Congenital anophthalmia, microphthalmia, dyslentia, open eyelid, congenital cataracts, and coloboma of the iris and retina are frequently encountered in C57BL/6J mouse fetuses exposed to physical, chemical, and nutritional stresses employed either transiently or continuously during pregnancy [42]. In searching the literature we noted a particularly strong similarity between our data and classical studies with prenatal exposure to ionizing radiation [41,43,44]. Over forty years ago, Hicks [43] observed that rat fetuses irradiated at the 2–8 somite pair stage of development were characterized almost exclusively by degrees of anophthalmia and microphthalmia resulting from widespread cell death throughout the embryonic head-fold. This observation was later confirmed in the C57BL mouse, where over 80 % of irradiated fetuses displayed ocular malformations [44]. Maximal sensitivity to radiation occurs during primary outgrowth of the optic vesicles and lens induction [43,44], which is the time frame of 2-CdA exposure used in our study. Therefore, we reproduced (with 2-CdA) a classical model in experimental teratogenesis (radiation-induced cell death) and advanced that model with evidence that susceptibility to induced eye defects, particularly of the lens, is directly related to the dosage of p53 protein activity in the embryo at the time of teratogen exposure.

Recent data have shown that *p53*-dependent apoptosis also disrupts the lens at later stages of eye development [21,45–47]. For example, an oncogenic growth signal related to systemic [45] or lens-specific [46,47] inactivation of the *Rb* tumor suppressor gene initiates *p53*-dependent apoptosis at the time of lens fiber differentiation, between days 12–13 pc, leading to dyslentia and microphthalmia. Transgenic mice expressing human *p53* under control of the lens-specific αA-crystallin gene promoter show excessive lens cell death and microphthalmia prior to birth, with the effect partially abrogated by the presence of mutant *p53* [21]. Our data indicate that p53-dependent apoptosis may have a dramatic effect on lens development even before primary lens fiber differentiation, as the cascade of teratogenic events from initiating mechanism to full manifestation of aphakia can take place between days 8–11 pc Apparently, the loss of even one functional *p53* allele can significantly 'harden' the lens primordium to apoptotic defects related to teratogenic insult or oncogenic growth signals.

A recent study proposed that *p53* functions as a teratological suppressor gene in response to DNA-damaging agents and developmental oxidative stresses [24]. In that study, $p53^{+/+}$ and $p53^{+/-}$ dams were bred to a $p53^{+/-}$ male, and given a mildly teratogenic regimen of dioxin on day 7 pc and benzo[a]pyrene on day 10 pc. Fetal resorptions were increased from 23 % to 44 % (primarily due to the death of $p53^{+/-}$ and $p53^{-/-}$ embryos), neonatal lethality was increased from 19 % to 74 %, and a spectrum of teratological anomalies was increased from 22 % to 47 % among litters derived from $p53^{+/+}$ versus $p53^{+/-}$ dams. The present data point to *p53* being required for apoptosis and teratogenesis. This is in provocative contrast with the study by Nicol *et al.* [24] and leads to the suggestion that *p53* plays a paradoxical role in teratogenesis — teratogenesis-suppressing on the one hand and teratogenesis-promoting on the other. Several factors might explain this paradox. One is the nature and degree of the environmental stress. For benzo[a]pyrene [24], the upstream initiating signal may require a build-up of oxidative damage related to teratogenic treatment, whereas the kind of lesion produced by 2-CdA is likely to be more acute. Thus, *p53* might normally confer a selective advantage to embryos under low-level oxidative stress whereas hyper-activation of *p53*-dependent events may initiate an array of cell death abnormalities the specificity of which depends on the amount of cell death, the stage of exposure, and the capacity for recovery.

Conclusions

Certain kinds of teratogens, exemplified here by the genotoxic agent 2-CdA, may produce their effects on developing embryos through *p53*-dependent events (apoptosis). Additional studies are needed to evaluate the generality of p53 induction as a biomarker for teratogenesis; however, results in the present study clearly point to the developing eye as a particularly vulnerable target organ system. This observation is consistent with several recent reports of *p53*-dependent apoptotic defects in lens differentiation following systemic or lens-specific induction by oncogenic growth signals [21,45–47]. In conclusion, *p53* represents a point of convergence for teratogenic or oncogenic lesions which may fashion dysmorphogenesis of a target organ field. In this context, wild-type *p53* may be an intrinsic genetic susceptibility factor for certain kinds of birth defects.

Materials and methods

Animals and treatment

TSG–p53 mice were purchased from GenPharm International (Mountainview, California). This strain harbors a replacement of exon 5 of the *p53* gene with a neo^R cassette, originally generated in 129/Sv-derived embryonic stem cells and backcrossed to C57BL/6J females [16]. The GenPharm colony, from Taconic Farms, is constantly maintained at the fourth backcross onto C57BL/6J through intercrossing of nullizygous males and heterozygous females; the wild-type control colony is also maintained at the fourth backcross and hence derives 93.75 % of its genetic background from C57BL/6J and the remainder from 129/Sv. Mice were kept on a 12 h photoperiod (0700–1900 h light). Virgin females ($p53^{+/+}$, $p53^{+/-}$) 3–5 months of age were bred to a male ($p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$) between 0830 to 1330 h and the presence

of a vaginal plug immediately afterwards signified coitus. For treatment with 2-CdA, dams received a single intraperitoneal injection of 0.2 ml per 30 g total body weight at 0930 h on day 8 pc. The 2-CdA was kindly provided by D. Carson. Stock solutions were prepared in sterile water and standardized by the millimolar extinction coefficient of 15 at 264 nm and diluted to deliver dosages as indicated; in some cases untreated litters provided the controls as the vehicle alone had no effect on any of the parameters tested.

Whole embryo culture

TSG–p53 embryos were harvested on day 8 pc (4–5 somite pairs, Theiler [40] stage 12) and cultured for 3 h in DME/F12 medium (Sigma Chemical Co., St. Louis, Missouri) containing 50 % (v/v) heat-deactivated human umbillical cord serum and 0.1 % (w/v) gentamicin sulfate. Because embryos were harvested from heterogenous TSG-p53 breeding pairs their genotypes were unknown to us during the experiment. Two embryos were placed in 1 ml culture medium in a 35 mm petri dish and incubated at 37 °C for 3 h in a conventional carbon dioxide incubator. Some were cultured in medium containing 5 μM 2-CdA; others were untreated controls. After culture, the visceral yolk sac was removed for allelotyping; the embryos were stained supravitally with 0.005 mg ml^{-1} AO and examined with a Nikon Optiphot fluorescence microscope under rhodamine optics [3]. The extent of AO-staining was rated based on comparison with uncultured specimens: type 1 (no change over uncultured specimens, demonstrating normal levels of programmed cell death in the head-fold); type 2 (small increase in AO-staining over normal amounts); and type 3 (large increase in AO-staining over normal amounts). Phenotypes were grouped by increasing severity (corresponding values of 1, 2, or 3) for statistical analysis.

Teratologic evaluation

Conceptuses from litters treated with 2-CdA on day 8 pc were evaluated on day 11 and day 17 pc. On day 11 pc the allelotype was determined from yolk sac DNA; embryos were fixed in neutral buffered formalin, processed for paraffin serial-sectioning, and stained with hematoxylin–eosin. On day 17 pc fetuses were examined grossly, weighed, fixed in 95 % ethanol, and stained with Alizarin red S for skeletal examination; allelotype was determined from skin or tail DNA samples obtained post-fixation. Individual eye phenotypes were rated [41] as: type 1 (normal), type 2 (colobomas of retina, narrowing of the pigment ring, abnormal pupillary diameter or shape); type 3 (moderate to severe microphthalmia); and type 4 (apparent anophthalmia). Individual eye phenotypes were grouped by severity (corresponding values of 1, 2, 3, or 4) for statistical analysis.

p53 allelotyping

Tissues were digested at 55 °C overnight in 50 mM Tris–HCl buffer (pH 8.5), containing 5 mM EDTA, 200 mM NaCl, 0.2 % sodium dodecyl sulfate and 0.2 mg ml^{-1} proteinase K. After purification, DNA was subjected to a multiplex PCR performed with 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μl *Taq* DNA Polymerase (GibcoBRL, Gaithersburg, Maryland) and primers: primer 2A (5′–GGGACAGCCAAGTCTGTTATGTGC–3′; mouse *p53* exon 4, forward), primer 2B (5′–CTGTCTTCCAGATACTCGGGATAC–3′; mouse *p53* exon 6, reverse), and primer 57 (5′–TTTACGGAGCCCTGGCGCTCGATGT–3′; *pol* II neomycin insert, reverse). After initial denaturation, PCR was performed for 35 cycles at 94 °C for 1 min, annealing at 62 °C for 2 min, extension at 72 °C for 6 min, autoextension for 10 sec, and final extension at 72 °C for 15 min. PCR products amplified from wild-type and targeted alleles (1.2 and 0.6 kilobases, respectively) were visualized on 1.5 % agarose gels with ethidium-bromide staining.

p53 immunostaining and TUNEL assay

Tissues were fixed overnight in phosphate-buffered formalin at 4 °C and processed for paraffin sectioning. Sections (optimally 5 μm thick) were dried onto positively-charged Superfrost/Plus microscope slides (Fisher Scientific). Immunoperoxidase staining for p53 protein employed CM5 polyclonal rabbit antiserum to recombinant murine p53 [36], kindly provided by P. Hall and D. Lane. Deparaffinated sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) and subjected to microwave retrieval at 95–100 °C for 10 min. After peroxidase block, CM5 antibody was applied at a dilution of 1:1000 (in 0.5 % bovine serum albumin–PBS) for 1 h at room temperature; nonimmune rabbit serum provided a negative control. Primary antiserum was localized with a Vectastain Elite ABC–peroxidase kit (Vector Laboratories, Burlingame, California). Color development employed the ImmunoPure metal enhanced peroxidase substrate kit (Pierce Chemical Company, Rockford, Illinois). Cell nuclei were scored as p53-positive if specific immunoperoxidase labeling was more intense than the corresponding cytoplasm.

Apoptotic DNA fragmentation was detected by *in situ* 3′ end labeling by the TUNEL method [37] using an ApopTag Kit (Oncor, Gaithersburg, Maryland). Because this method can also detect drug-induced DNA damage [29], we assumed that teratogenic treatment with 2-CdA would induce TUNEL labeling that was independent of *p53* gene background, and not obviously associated with necrotic or apoptotic (that is, AO-positive) cells. After deparaffination, sections were digested for 15 min at room temperature with proteinase K in PBS. The concentration of proteinase K was critical; we routinely tested several proteinase concentrations in all TUNEL specimens. Proteinase K concentration was titrated to a level yielding optimal specificity for clearly dying cells (generally 2–4 μg ml^{-1}) which is well below the concentration recommended in the ApopTag protocol (20 μg ml^{-1}). After peroxidase block, free 3′-ends of DNA fragments were labeled with terminal deoxynucleotidyl transferase. Negative controls included substitution of water for terminal deoxynucleotidyl transferase in the reaction buffer. Color development employed the ImmunoPure substrate. Sections were counterstained with 0.5 % (w/v) methyl green in 0.1 M sodium acetate (pH 4.0), for 6 min and differentiated in water and butanol.

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